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# Kinetic studies of the reaction of hydrogen peroxide with manganese-reconstituted horseradish peroxidase

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The kinetics of the transient reaction of hydrogen peroxide with manganese-reconstituted horseradish peroxidase (MnHRP) has been studied using stopped-flow spectrophotometry. The specificity of the reaction seems to be maximal at physiological pH. The bell-shaped pH dependence of the formation of the resulting 'peroxide' compound I has been interpreted in terms of two ionisable groups at the active site of the enzyme; the $pK_a$ of one group was found to be near 4.8 and that of the other near 10.6. Values of the apparent second-order rate constant determined at various temperatures in the range 20–50 °C were used to calculate the thermodynamic parameters of the reaction. The apparent activation energy for the formation of MnHRP compound I was found to be higher than that of the native peroxidase and the value of the minimum energy of activation in water ($E^{\ddagger}_{H_2O}$), indicating that the enthalpy change associated with the binding of MnHRP to $H_2O_2$ may be positive.

Horseradish peroxidase (E.C. 1.11.1.7, donor-$H_2O_2$ oxidoreductase; HRP) is a haem protein which catalyses the oxidation of various substrates by hydrogen peroxide. The mechanism involves electron transfer from the metal centre of the haem group to $H_2O_2$, resulting in the formation of intermediate peroxide compounds.[1–7] Horseradish peroxidase forms peroxide compound I (HRP-I) by donating two reducing equivalents to $H_2O_2$. During the catalytic turnover, HRP-I reacts with organic substrates to give HRP-II. The mechanism of formation of these peroxide compounds has long been a subject of interest.[8] The existence of the intermediate peroxide compound(s) of the haem proteins (which control the protein functions) is regulated by the central metal atom and the architecture of the protein cavity surrounding the haem prosthetic group. To understand the role of metal ions in the formation of the peroxide compounds, studies have been performed on metal-reconstituted proteins.[8–10] Manganese protoporphyrin-reconstituted HRP (MnHRP) has been shown to form one peroxide compound on reaction with $H_2O_2$.[9] Nick *et al.*[10] have discussed the formation of the peroxide compound of MnHRP which contains a weak Mn=O bond and a protein-based non-porphyrin radical. Further, MnHRP has been shown to exhibit lower peroxidase activity in comparison to that of native HRP.[9]

The present paper reports a kinetic investigation of the reaction of MnHRP with $H_2O_2$. To understand the nature of the enzyme's active site experiments were performed at various pH. The results on the formation of MnHRP compound I indicate the involvement of two pH-dependent equilibria of the amino acid residues near the prosthetic group of MnHRP. The corresponding apparent thermodynamic activation parameters have been determined from studies carried out at different temperatures.

## Experimental

Protoporphyrin IX dimethyl ester (dimethyl 3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoate) and salt-free lyophilised powder of HRP were obtained from Sigma Chemical Co. Hydrogen peroxide used was of reagent grade. Crude HRP was purified by ion-exchange chromatography on CM52 cellulose. Its purity was checked from its Rz ($A_{403}/A_{280} = 3.2$) value.[11]

Manganese(III) protoporphyrin IX was synthesised by the reported method[9,12] and purified on a Celite column.[13] The horseradish apoperoxidase was prepared by a slight modification of Teale's acid–butanone procedure.[14] An aqueous solution of HRP (0.5 mmol $dm^{-3}$) in 10 mmol $dm^{-3}$ sodium dihydrogen phosphate was adjusted to pH 2.3–2.4 by addition of dilute ice-cold hydrochloric acid, at 0 °C. The mixture was immediately mixed with an equal volume of ice-cold butan-2-one, shaken for 30 s and allowed to settle for 5 min. The upper butanone layer was siphoned off, and the remaining aqueous phase treated several times with ice-cold butan-2-one until the butanone phase became colourless. The aqueous mixture was dialysed at 4 °C against 10 mmol $dm^{-3}$ $NaHCO_3$, then water, and finally against 0.01 mol $dm^{-3}$ tris(hydroxymethyl)-aminomethane (Tris)–HCl buffer, pH 8.0. The concentration of horseradish apoperoxidase was measured and found to be in the range 0.2–0.3 mmol $dm^{-3}$.

Manganese(III) protoporphyrin IX reconstituted horseradish peroxidase was prepared according to the procedure of Yonetani and Asakura.[9,15,16] The apoperoxidase (0.2 mmol $dm^{-3}$) in 0.01 mol $dm^{-3}$ Tris–HCl, pH 8.0 was mixed with a 1.1-fold excess of manganese(III) protoporphyrin IX, shaken thoroughly and allowed to stand for 10 min. The mixture was adjusted to pH 7.0 and loaded onto a DEAE-cellulose column preequilibrated with 10 mmol $dm^{-3}$ $NaH_2PO_4$ buffer, pH 7.0. Manganese(III) protoporphyrin IX remained firmly bound to the column and $Mn^{III}$HRP was eluted, concentrated using an ultrafiltration cell, lyophilised and stored at −37 °C.

The UV/VIS absorption spectra were recorded with a Shimadzu UV-2100 spectrophotometer using a cell with 1 cm path length. The kinetic measurements were performed on a microprocessor-controlled stop-flow machine (HITECH-500) by observing the increase in absorbance at 412.5 nm due to the formation of the peroxide. The concentration of $H_2O_2$ was measured from the absorbance at 203 nm ($\varepsilon = 72.4$ $dm^3$ $mol^{-1}$ $cm^{-1}$)[12,17] and was varied in the range 2.5–35 mmol $dm^{-3}$. The buffers used were acetate (pH < 5.9), phosphate (6.0–8.0), Tris (8.0–9.0) and carbonate (9.3–10.6). The buffer concentration was constant at 50 mmol $dm^{-3}$ and ionic strength was adjusted with potassium nitrate to 0.2 mol $dm^{-3}$. The concentration of $Mn^{III}$HRP was kept constant at 2.5 μmol $dm^{-3}$. Under these conditions the reaction followed a pseudo-first-order rate law.

## Results

Manganese protoporphyrin IX-reconstituted HRP (MnHRP) was observed to form MnHRP compound I on quantitative

addition of hydrogen peroxide. Fig. 1 (inset) shows the optical spectra of native and hydrogen peroxide-treated MnHRP, which match with the spectra reported by others.[9,10] In order to understand the mechanism of the formation of MnHRP compound I, we have performed a stopped-flow investigation of the transient reaction of MnHRP with $H_2O_2$. Fig. 1 shows a typical stopped-flow trace showing the monophasic increase in absorbance at 413 nm due to the formation of MnHRP compound I. The corresponding pseudo-first-order rate constant ($k_{obs}$) was calculated from the single exponential fit of the experimental data, *e.g.* $k_{obs}$ 43 $s^{-1}$ at pH 6.8 and 25 mmol $dm^{-3}$ $H_2O_2$.

Yonetani and Asakura[9] have studied the oxidation of ferrocytochrome c by $H_2O_2$ catalysed by MnHRP, and proposed the existence of a $H_2O_2$-bound MnHRP complex. Considering a similar kind of mechanism, the transient reaction of MnHRP with $H_2O_2$ can be expressed as in equation (1). This leads

$$\text{MnHRP} + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{MnHRP–}H_2O_2] \xrightarrow{k_2} \text{MnHRP-I} \quad (1)$$

to a dependence of $k_{obs}$ on $[H_2O_2]$,[18,19] equation (2), where

$$\frac{1}{k_{obs}} = \frac{K_D}{k_2}\frac{1}{[H_2O_2]} + \frac{1}{k_2} \quad (2)$$

$K_D$ ($= k_{-1}/k_1$) is the dissociation constant of the enzyme–substrate complex and $k_2$ the rate of formation of the product from this complex, *i.e.* the turnover number of the reaction.

The formation of MnHRP compound I was monitored at various concentrations of $H_2O_2$ and the corresponding values of $k_{obs}$ were determined. A plot of $k_{obs}$ *vs.* $[H_2O_2]$ at pH 6.8 is shown in Fig. 2. It is consistent with equation (3) which is

$$k_{obs} = k_{app}[H_2O_2] \quad (3)$$

obtained from (2) under the condition that $K_D \gg [H_2O_2]_0$. The $k_{app}$ ($=k_2/K_D$) value for the formation of MnHRP compound I was found to be $1.7 \times 10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$ at pH 6.8.

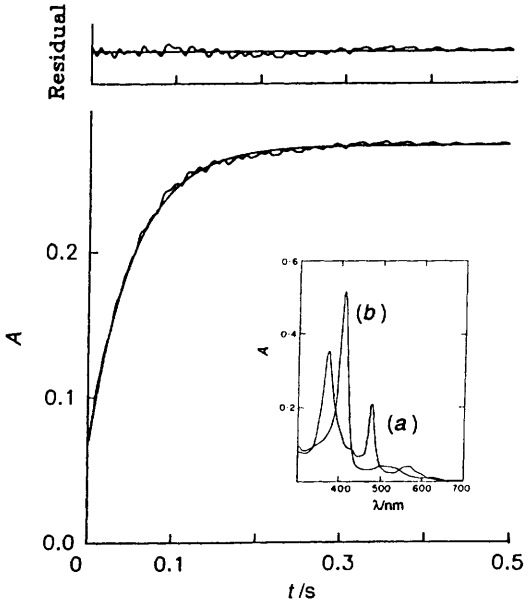



**Fig. 1** Typical stopped-flow trace for the reaction of MnHRP (2.5 μmol $dm^{-3}$) with $H_2O_2$ (12.5 mmol $dm^{-3}$) at pH 6.8 showing the increase in absorbance at 412.5 nm. The line drawn through the experimental trace is a computer fit using a single exponential function. The residual ($A_{obs} - A_{calc}$) plot shows the accuracy of the computer-generated fit. Inset: the optical spectra of MnHRP (*a*) and MnHRP compound I (*b*) in 50 mmol $dm^{-3}$ phosphate buffer (pH 7.0); 10 mmol $dm^{-3}$ $H_2O_2$ was added to 5 μmol $dm^{-3}$ MnHRP to obtain compound I

To examine the influence of hydrogen ions on MnHRP compound I, we have studied the kinetics of the transient reaction of MnHRP with $H_2O_2$ at various pH. The corresponding values of $k_{app}$ were determined with the help of equation (3). Fig. 3 shows a typical bell-shaped pH dependence of $k_{app}$ which indicates that at least two ionisable groups affect the rate of formation of MnHRP compound I by $H_2O_2$.

### Determination of thermodynamic activation parameters

The temperature dependence of the transient reaction can be used to calculate the apparent enthalpy ($\Delta H^{\ddagger}$), free energy ($\Delta G^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) of activation using equations (4)–(6)

$$\Delta H^{\ddagger}_{app} = E^{\ddagger}_{app} + RT \quad (4)$$

$$k_{app} = \frac{k_B T}{h}\exp\left(\frac{-\Delta G^{\ddagger}_{app}}{RT}\right) \quad (5)$$

$$k_{app} = \frac{k_B T}{h}\exp\left(\frac{\Delta S^{\ddagger}_{app}}{R}\right)\exp\left(\frac{-\Delta H^{\ddagger}_{app}}{RT}\right) \quad (6)$$

where $k_B$ and $h$ are the Boltzmann and Planck's constants,

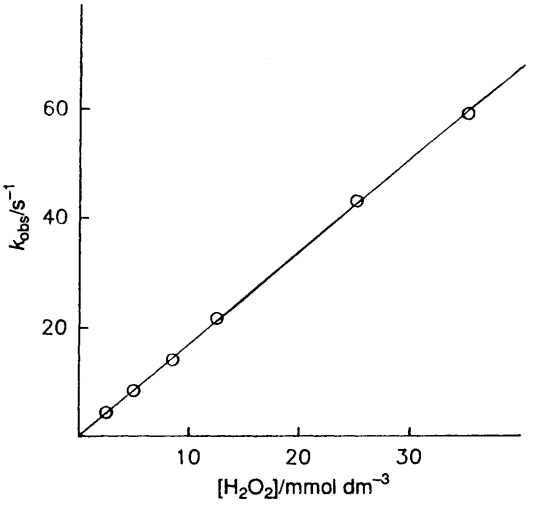



**Fig. 2** Plot of the observed pseudo-first-order rate constant ($k_{obs}$) as a function of hydrogen peroxide concentration at pH 6.8 and 25 °C. The concentration of MnHRP was kept constant at 2.5 μmol $dm^{-3}$. The slope of the line corresponds to $k_{app}$ the second-order rate constant, and the intercept was found to be zero

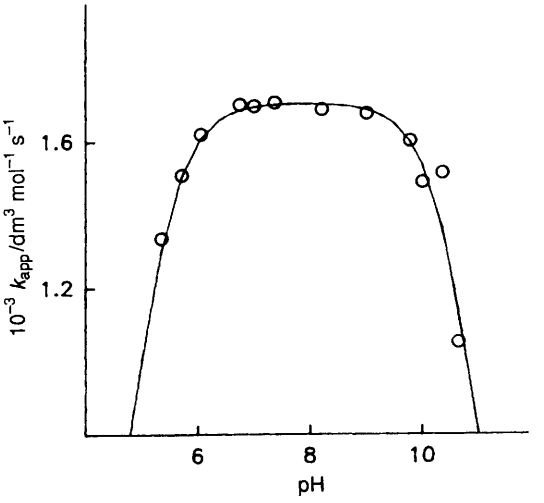



**Fig. 3** Plot of $k_{app}$ as a function of pH at 25 °C. The solid line through the experimental points is given by equation (7)

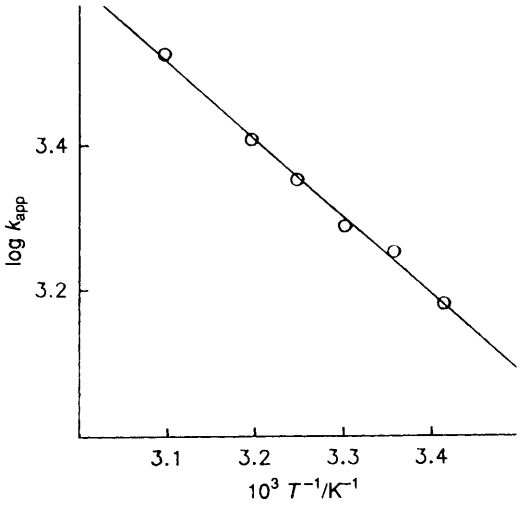


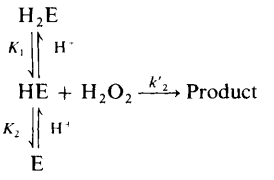

**Fig. 4** Arrhenius plot for the formation of MnHRP compound I from MnHRP and $H_2O_2$ at 50 mmol $dm^{-3}$ phosphate, pH 7.0

respectively. The formation of the peroxide compound was monitored at various temperatures in the range 20–50 °C and the Arrhenius plot for $k_{app}$ is shown in Fig. 4. The activation energy for the formation of MnHRP compound I was determined from the slope as $E^{\ddagger}_{app} = 20.41 \pm 0.69$ kJ $mol^{-1}$. The data from these experiments were used to calculate the apparent thermodynamic activation parameters [see equations (4)–(6)]: $\Delta G^{\ddagger}_{app} = 54.44 \pm 1.85$ kJ $mol^{-1}$, $\Delta S^{\ddagger}_{app} = -105.86 \pm 3.6$ J $K^{-1}$ $mol^{-1}$, and $\Delta H^{\ddagger}_{app} = 22.89 \pm 0.78$ kJ mol at 25 °C.

## Discussion

The value of $k_{app}$ for the reaction of $H_2O_2$ with MnHRP obtained here is $1.7 \times 10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$, similar to the corresponding value ($8.4 \times 10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$) for manganese mesoporphyrin IX (7,12-diethyl-3,8,13,17-tetramethylporphyrin-2,18-dipropanoate) reconstituted HRP.[9] Thus, the changes in substitution of the porphyrin ring in MnHRP does not seem to have a significant effect on the formation of MnHRP compound I. The $k_{app}$ for the formation of peroxide compounds of manganese-reconstituted cytochrome c peroxidase (MnCcP) was, however, reported to be $7.5 \times 10^5$ $dm^3$ $mol^{-1}$ $s^{-1}$;[9] the values for native HRP and CcP are $1.8 \times 10^7$ and $1.4 \times 10^8$ $dm^3$ $mol^{-1}$ $s^{-1}$ respectively.[8,20] Comparison of the $k_{app}$ values for the native and manganese-reconstituted proteins indicates a large decrease in the apparent formation rate due to metal substitution in both HRP and CcP.

The apparent second-order rate constant ($k_{app}$) is related to $k_{cat}/K_M$ (*i.e.* $k_{app} = k_{cat}/K_M$), where $k_{cat}$ and $K_M$ are the turnover number and Michaelis constant respectively.[19] This ratio denotes the specificity of a reaction and its magnitude is related to the properties of the free enzyme and free substrate. Thus, the bell-shaped pH dependence of $k_{app}$ indicates that the specificity of $H_2O_2$ towards MnHRP is maximal near physiological pH. This dependence for the formation of MnHRP compound I is in contrast to that for the formation of native HRP compound I, which is basically independent of pH above pH 5.[21] This indicates that there are two ionisable groups on the enzyme which affect the rate of reaction of hydrogen peroxide with MnHRP, and the rate of reaction is maximal when the group with the acid dissociation constant $K_1$ is deprotonated and that with the acid dissociation constant $K_2$ is protonated. The simplest mechanism consistent with the pH dependence of $k_{app}$ is given by Scheme 1 where E, HE and $H_2E$ are deprotonated, protonated and doubly protonated species of the free enzyme. The charges on the different species have been omitted for simplicity. Equation (7) can be derived from Scheme 1,[22] where




**Scheme 1**

$$k_{app} = \frac{k'_2}{([H^+]/K_1) + K_2[H^+]^{-1} + 1} \quad (7)$$

$k'_2$ is the pH-independent second-order rate constant and $K_1$ and $K_2$ are the equilibrium constants for amino acid residues of the proteins near the prosthetic group (see Fig. 3). The fitted values of $pK_1$ and $pK_2$ were 4.82 ± 0.06 and 10.56 ± 0.52 respectively and the value of $k'_2$, which is independent of pH, was $1.78 \times 10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$.

The pH dependence of the formation of various compound I, *viz.* those of horseradish peroxidase,[23] lactoperoxidase,[24] human myeloperoxidase,[25] lignin peroxidase,[26] bromoperoxidase,[27] turnip peroxidase[28] and intestinal peroxidase,[29] has indicated that peroxidases possess a distal ionisable group, the protonation state of which controls the rate of formation of compound I. The p$K$ value of this group always seems to be in the range 3.0–5.3. The observation of one $pK_a$ in this range suggests that the distal group which influences the formation of compound I in other peroxidases, more specifically in HRP, might also be responsible for influencing the formation of MnHRP compound I. In case of HRP, His-42 has been proposed to be the distal group.[30]

The reaction of HRP-II with $H_2O_2$ has also been reported to show a bell-shaped pH dependence, which was explained by a similar kind of mechanism to that described above, involving the ionisation of two groups having $pK_1$ 4.2 ± 0.1 and $pK_2$ 9.1 ± 0.3.[22] The oxidation of *p*-cresol by HRP compound II has been shown to be influenced by two groups having $pK_a$ 5.4 and 8.6.[31] These observations suggest that the groups influencing the reactions of $H_2O_2$ with MnHRP and with HRP-II may be the same. The small change in p$K$ may be due to some change in Lewis acid character of the central metal and/or to the change in hydrophobicity of the active-site protein cavity.

The apparent activation energy for the formation of compound I of native HRP has been reported to be 14.64 ± 4.18 kJ $mol^{-1}$.[32] That (20.41 ± 0.69 kJ $mol^{-1}$) for the formation of MnHRP compound I, is higher. This is not unexpected since MnHRP is not really a pure enzymatic species and the expected energy of activation of MnHRP should be more than that of native HRP. The apparent activation energy involves a contribution from the $H_2O_2$ binding step as well as one from the subsequent formation of compound I.[33,34] Since $k_{app}$ for the formation of MnHRP compound I involves $k_2$ and $K_D$ [see equation (3)], $E^{\ddagger}_{app}$ can be expressed as in equation (8)

$$E^{\ddagger}_{app} = E^{\ddagger}_{k_2} + \Delta H_0 \quad (8)$$

where $E^{\ddagger}_{k_2}$ is the activation energy for the formation of MnHRP compound I from MnHRP–$H_2O_2$ and $\Delta H_0$ is the enthalpy change associated with the binding of $H_2O_2$ to MnHRP. A lower value of $E^{\ddagger}_{app}$ compared to $E^{\ddagger}_{H_2O}$ has been observed for electron-transfer reactions between certain metal complexes which associate in solution prior to a redox step because the values of $\Delta H_0$ were negative.[35] The low value of $E^{\ddagger}_{app}$ for the formation of compound I from various peroxidases and catalases in water has been explained on a similar basis.[34,36] It has not been possible to determine the individual values of $E^{\ddagger}_{k_2}$ and $\Delta H_0$ for the reaction of MnHRP with $H_2O_2$. However, the observation that the activation energy for the formation of MnHRP compound I is higher than

$E^{\ddagger}_{H_2O}$ (20.41 ± 0.69 as compared to 16.28 ± 0.17 kJ $mol^{-1}$) indicates that the enthalpy change associated with the binding of MnHRP with $H_2O_2$ may be positive.

We have determined apparent thermodynamic activation parameters for the formation of MnHRP compound I: $\Delta H^{\ddagger}_{app}$ is positive, however, $\Delta S^{\ddagger}_{app}$ is negative. The positive value of $\Delta H^{\ddagger}_{app}$ indicates that the formation of MnHRP compound I is endothermic. Since $\Delta H^{\ddagger}_{app} \ll T\Delta S^{\ddagger}_{app}$, it is suggested that the free-energy change, $\Delta G^{\ddagger}_{app}$, is dominated by $\Delta S^{\ddagger}_{app}$. The negative sign of the entropy can be explained on the basis that the reaction between MnHRP and $H_2O_2$ (which is basically a neutral substrate) results in electron redistribution as a result of which the activated complex will be more polar than the reactants. There is, therefore, an increase in restriction of movement of electrons (an increase in electrostriction) and a corresponding negative activation entropy.[37]

Job *et al.*[34] have compared the enthalpies and entropies of formation of compound I from several haem-containing proteins and a haem complex. They observed that the rate constants for the reaction of $H_2O_2$ with the non-enzymatic species (metal porphyrin and different types of haemoglobin and myoglobin, which do not act as an enzyme) are of the order of $10^4$ $dm^3$ $mol^{-1}$ $s^{-1}$ or less, and $10^7$ $dm^3$ $mol^{-1}$ $s^{-1}$ for the peroxidases and catalases. The activation enthalpies for the non-enzymatic species are found to be in excess of that for the ionisation of hydrogen peroxide (34.43 kJ $mol^{-1}$), and the activation entropies scattered around a value of zero. Such values have been explained on the basis that the non-enzymatic species are incapable of reacting with $H_2O_2$. The activation enthalpies for the peroxidases and catalases are less than that for a diffusion-controlled reaction and much less than that of the enthalpy of ionisation of hydrogen peroxide. Hence, it appears that the non-enzymatic species react with the strong nucleophile $HO_2^-$. Our observation that the rate constant for the reaction of MnHRP with $H_2O_2$ is of the order of $10^3$ $dm^3$ $mol^{-1}$ $s^{-1}$ places MnHRP in the category of the non-enzymatic species. However, the activation enthalpy for the present reaction (22.89 kJ $mol^{-1}$) is much less than that of the enthalpy of ionisation of hydrogen peroxide. This may indicate that it is hydrogen peroxide which is reacting with MnHRP.

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